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ORIGINAL ARTICLE

Analysis of catecholamines and related compounds in one whole metabolic pathway with high performance liquid chromatography based on derivatization

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High performance liquid chromatography;
Fluorescence detection

Abstract The simultaneous determination of Tyr, catecholamines and their metabolites in one whole metabolic pathway using high performance liquid chromatography coupled with fluorescence detection (HPLC–FLD) based on pre-column derivatization has been achieved successfully. 1,3,5,7-Tetramethyl-8-(*N*-hydroxysuccinimidyl butyric ester)-difluoroboradiaza-*s*-indacene (TMBB-Su), a highly reactive fluorescent reagent synthesized in our previous work, was used for the labeling of tyrosine (Tyr), L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), norepinephrine (NE), epinephrine (E) and metanephrine (MN). Derivatization conditions including reagent concentration, buffer, reaction temperature and reaction time were also investigated to improve the derivatization efficiency and thus the sensitivity of the detection. The separation of the derivatives was obtained on C_{18} column with the mobile phase of 20 mM pH 3.5 citric acid (H_3Cit)–sodium hydrogen phosphate (Na_2HPO_4) buffer and methanol. Good linearities with correlation coefficients square (R^2) greater than 0.998 in the corresponding concentration ranges were observed and the detection limits ($S/N = 3$) were found in the range from 0.10 to 0.40 nM (L-DOPA: 1.45 nM). The proposed method has been applied to the detection of catecholamines and related compounds in mice liver and brain samples without tedious extraction or purification procedure, which exhibits excellent selectivity and sensitivity in the analysis of complex samples. This work provides an alternative approach in the metabolic research of catecholamines and is helpful for the study of catecholamine metabolism.

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1. Introduction

Catecholamines are important neurotransmitters mainly including dopamine (DA), norepinephrine (NE) and epinephrine (E), which play crucial roles in the regulation of nervous and cardiovascular systems (Gu et al., 2008; Kim et al., 2008; Sakaguchi et al., 2011) and are involved in some brain behaviors such as stress, panic, anxiety and depression (Parrot et al., 2011; Yoshitake et al., 2003). Changes of catecholamines concentrations in organisms have a close connection with some neurological disorders (Raggi et al., 1999) and certain diseases. Furthermore, there are many metabolites that are derived from tyrosine (Tyr) in the whole metabolome of catecholamines (Bicker et al., 2013), and the determination of these metabolites can provide unique information about disease development and treatment effects when measured in combination with catecholamines neurotransmitters. For instance, L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of DA, is used as an effective medical therapy of Parkinson's disease resulting from lack of dopamine for it can be converted to dopamine easily by decarboxylation (Frey, 2001; Törnkvist et al., 2004). The measurement of normetanephrine (NMN) and metanephrine (MN), metabolites of NE and E respectively, is a useful screening method for pheochromocytomas (Eisenhofer et al., 2011, 1999; Lagerstedt et al., 2004). Therefore, the quantitative determination of a series of metabolites in one metabolic pathway of catecholamines in biological samples is of great significance, which is helpful in finding out biomarkers associated with related diseases.

High performance liquid chromatography (HPLC) with fluorescence detection (FLD) is a powerful method widely used for the analysis of trace bioactive molecules in a complex matrix because of its good sensitivity, high selectivity and simple sample pretreatment. Catecholamines and related compounds have intrinsic detectable fluorescence and the corresponding HPLC methods have been reported (Honma et al., 1987; Said et al., 1990; Singh et al., 2010; Wood and Hall, 2000). However, the short detection wavelengths and the alternation of the wavelengths between different metabolites limit their practical applications. Besides, the detection sensitivity based on intrinsic fluorescence is always not satisfactory. Since catecholamines and some related compounds have the amino group, amine-reactive fluorescent reagents have been naturally used in their detection with HPLC, which can afford high sensitivity. These reagents include naphthalene-2,3-dicarboxaldehyde (NDA) (Kawasaki et al., 1989), *o*-phthalaldehyde (OPA) (Zhao et al., 2011), fluorescamine (Díaz et al., 2009), 9-fluorenylmethyloxycarbonyl chloride (FMOC–Cl) (Chan et al., 2000), *N*-hydroxysuccinimidyl-3-indolylacetate (SIIA) (Wang et al., 1999), *N*-hydroxysuccinimidyl fluorescein-*O*-acetate (SIFA) (Wang et al., 2000) and so on. Besides, some reagents which can react with hydroxyl groups of catechols are also reported for the determination of catecholamines. For instance, 1,2-diphenylethylenediamine (DPE) can achieve high sensitivity of the detection of analytes through reacting with catechols (Fujino et al., 2003; Liu et al., 2011; Muzzi et al., 2008), but catechols have to be oxidized to the corresponding quinones in the presence of potassium hexacyanoferrate (III) before reacting with DPE. Although these fluorescent reagents have been successfully employed in the detection of catecholamine neurotransmitters and some related metabolites, the simultaneous determination of Tyr, catecholamines and their metabolites in one whole metabolic pathway of catecholamines is few.

1,3,5,7-Tetramethyl-8-(*N*-hydroxysuccinimidyl butyric ester)-difluoroboradiaza-*s*-indacene (TMBB-Su) is a highly sensitive, difluoroboraindacene (BODIPY)-based labeling reagent for amino compounds developed in our previous work. Research had shown that TMBB-Su had high fluorescence quantum yield, excellent reactivity and good selectivity for amino compounds (Gao et al., 2011; Zhang et al., 2011). Moreover, TMBB-Su can react with both primary and secondary amines as well as amino acids, and the formed derivatives are stable enough, which means TMBB-Su is suitable for the analysis of metabolism of amino compounds. To validate the potential of TMBB-Su in the study of catecholamines metabolism and even metabonomics research, a HPLC-FLD method using TMBB-Su as a pre-column labeling reagent for the metabolic analysis of catecholamines has been developed by selecting one whole metabolic pathway of catecholamines as representative (as shown in Fig. 1), including Tyr, L-DOPA, DA, NE, E and MN. And the proposed method has been successfully applied to the analysis of catecholamines and related compounds in one whole metabolic pathway in mice liver and brain samples, which demonstrates that the HPLC–FLD method based on derivatization has great potential in the metabolic analysis of catecholamines.

2. Experimental

2.1. Apparatus

Experiments were performed using an LC-10A HPLC system (Shimadzu, Tokyo, Japan) with Shimadzu LC-10AD dual-pump, RF-10AXL fluorescence detector, a CTO-10A column oven and an N2000 chromatography data system (Zhejiang University, Hangzhou, China). Samples were injected manually using a 20-μL sample loop. The separation was performed on a C_{18} column (5 μm, 250 mm × 4.6 mm id, Kromasil, Bohus, Sweden). The pH value of the solution was measured using a Mettler Toledo (Shanghai, China) Delta 320 meter.

2.2. Chemicals and reagents

Tyr, L-DOPA, DA, NE, E and MN were purchased from Sigma (St. Louis, MO, USA). TMBB-Su was synthesized in our lab. All aqueous solutions were prepared using water purified by a Milli-Q gradient system (Millipore, Bedford, MA, USA). Unless stated otherwise, all other reagents used were of analytical grade.

The stock solution of TMBB-Su (5.0×10^{-3} M) was prepared by dissolving TMBB-Su in anhydrous acetonitrile. The stock solutions (1.0×10^{-3} M) of the analytes (Tyr, L-DOPA, DA, NE, E and MN) were prepared in water or 0.01 M hydrochloric acid (HCl), and diluted with water to the desired concentrations before use. When not in use, all standard solutions were stored at −30 °C. Boric acid (H_3BO_3)–sodium tetraborate ($Na_2B_4O_7$) buffer was prepared by mixing 0.05 M $Na_2B_4O_7$ solution with 0.2 M H_3BO_3 solution to the required pH value. Citric acid (H_3Cit)–sodium hydrogen phosphate (Na_2HPO_4) buffer was prepared by mixing 0.1 M H_3Cit solution with 0.1 M Na_2HPO_4 solution to the required pH value.

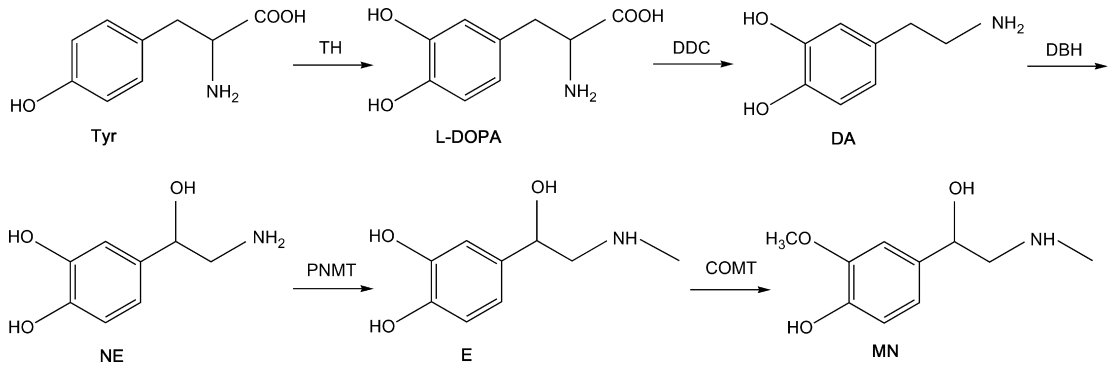

Figure 1 Biosynthesis and metabolism of catecholamines. TH: tyrosine hydroxylase; DDC: dopa decarboxylase; DBH: dopamine β-hydroxylase; PNMT: phenolethanolamine-*N*-methyltransferase; COMT: catechol-*O*-methyltransferase.

2.3. Synthesis of TMBB-Su

TMBB-Su was synthesized according to the synthesis method developed in our previous work (Zhang et al., 2011), which was described as follows. Firstly, 1,3,5,7-tetramethyl-8-butyric acid difluoroboradiaza-*s*-indacene (1) was synthesized with reference to a literature method (Li et al., 2006). Then, a mixed solution of the product (1), *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in anhydrous tetrahydrofuran (THF) was stirred for 10 h at room temperature and the mixture was filtered to remove the precipitate. After the residue was purified, concentrated and dried, a red product, 1,3,5,7-tetramethyl-8-*(N*-hydroxysuccinimidyl butyric ester)-difluoroboradiaza-*s*-indacene, was obtained.

2.4. Derivatization procedure and analysis

The derivatization of TMBB-Su with amino compounds was completed in alkaline solution. 175 μL of mixed solution of catecholamines and related compounds was placed in 1.5-mL Eppendorf vial, and then 65 μL of H_3BO_3–$Na_2B_4O_7$ buffer (pH 7.6) and 65 μL of TMBB-Su (5.0×10^{-3} M) were added successively. The mixture was diluted to 500 μL with acetonitrile. The vial was capped tightly and kept at 25 °C for 40 min. After dilution with the mobile phase, an aliquot (20 μL) of the reaction mixture was injected directly into the liquid chromatographic system. The reagent blanks without mixed catecholamines and related compounds were also prepared in the same procedure.

2.5. Chromatographic separation

Reversed phase high performance liquid chromatography was used for the separation of derivatives of TMBB-Su and analytes. To get better separation efficiency, binary gradient elution was used on Kromasil C_{18} column. Eluent A was methanol and eluent B was 20 mM pH 3.5 H_3Cit–Na_2HPO_4 buffer solution. Before the analysis, the C_{18} column was pre-equilibrated with the mobile phase of 58% A (v/v) for 30 min. The gradient elution condition began with an isocratic elution of 58% A (v/v) for 30 min, followed by a gradual increase of A from 65% to 100% (v/v), until 42 min. Finally, the mobile phase was reset (A:B) 58:42 (v/v) and stayed for 5 min to equilibrate for the next injection. The flow rate was set at 0.7 mL/min and the column temperature was kept at 22 °C. The detection wavelengths were 490 nm and 510 nm for excitation and emission, respectively.

2.6. Sample preparation

Male Kunming mice were obtained from the Center for Animal Experiment/ABSL-3 Laboratory of Wuhan University (Wuhan, China). The mice were housed at room temperature, with free access to food and water. All experiments with live animals in this report were performed according to the principles of laboratory animal care and Chinese national law. Mice were sacrificed by cervical dislocation on an ice bag, then the liver and brain tissues were collected and frozen immediately. The pretreatment of samples was performed according to the references method (Parrot et al., 2011; Vaarmann et al., 2002; Yoshitake et al., 2004; Zhao and Suo, 2008). About 0.2 g tissues each were dissected and homogenized in 10 μL of ice–cold 0.1 mol/L perchloric acid per mg of tissue respectively. The homogenate was transferred into the centrifuge tube, sonicated with an ultrasonicator, vortexed and centrifuged (8000 rpm for 15 min) at 4 °C. Analytes were extracted into the supernatant. And then 1 mL of the supernatant was neutralized with 50 μL of 1 M sodium hydroxide (NaOH) solution. The supernatant was diluted 10 times further with water and derivatized directly with TMBB-Su.

2.7. Method validation procedure

2.7.1. Linearity

The linearity of the method was determined by the analysis of a mixture of six target compounds with seven different concentrations from 0.004 to 0.2 μM for L-DOPA, from 0.002 to 0.5 μM for Tyr and DA, from 0.002 to 1 μM for NE and E and from 0.002 to 0.2 μM for MN, respectively. Calibrations plots generated by plotting the peak area versus the concentration were subjected to calculate the regression equations and correlation coefficients (*R*).

2.7.2. Precision

The precision of the method expressed as the relative standard deviations (RSDs) includes intra-day precision and inter-day precision. The precision of the method was determined from three concentration levels (0.1 μM for Tyr, DA, NE, E and

MN and 0.2 μM for L-DOPA; 0.05 μM for Tyr, DA, NE, E and MN and 0.1 μM for L-DOPA; 0.01 μM for Tyr, DA, NE, E and MN and 0.02 μM for L-DOPA) by repeating six sequential runs ($n = 6$) for each concentration on the same day and two consecutive days respectively using the standard solutions.

2.7.3. Limit of detection

Limit of detection was determined by assaying the standard solutions at gradually decreased concentrations until the lowest concentration that provided a peak area with a signal-to-noise ratio of 3.

2.7.4. Accuracy

The accuracy of the method was estimated by the standard addition method. In the proposed method, the accuracy was determined by adding three concentration levels (0.025 μM for Tyr, DA, NE, E and MN and 0.05 μM for L-DOPA; 0.05 μM for Tyr, DA, NE, E and MN and 0.10 μM for L-DOPA; 0.07 μM for Tyr, DA, NE, E and MN and 0.14 μM for L-DOPA, respectively) into the samples. Recovery was calculated from the added concentrations and the practically found concentrations.

3. Results and discussion

3.1. Optimization of separation condition to improve the selectivity

The analytes of this study are catecholamines and related compounds, and their structures are similar to each other. Thus, the polarities and solubilities of their derivatives after derivatization with TMBB-Su both only have slight distinction, and the separation is an obvious challenge.

Initially, isocratic elution mode was selected for the separation and the effect of methanol content was investigated first in the range of 55–75%. When the methanol content was higher than 65%, the peaks of Tyr, L-DOPA and DA derivatives had no good resolution. While the methanol content was less than 55%, the peaks tended to be separated, but the separation time was long and peaks were wide. Better separation efficiency and shorter separation time were obtained within the methanol content range of 55–60%. Finally, 58% methanol was used in this experiment.

After derivatization with TMBB-Su, catecholamines and related compounds have one or two phenolic hydroxyl groups. The ionization states of these phenolic hydroxyl groups can be affected by the pH value of the mobile phase, and thus the retention time of these analytes also could be altered with the change of pH value. Therefore, pH value of the mobile phase is a key factor to the separation in this study. H_3Cit–Na_2HPO_4 buffer was used for adjusting the pH value of the mobile phase ranging from 2.0 to 7.0. The peak of E derivative overlapped with that of TMBB-Su hydrolyzate if the buffer pH was below 3.0, and the peak of Tyr derivative could not be resolved when the pH was higher than 4.0. However, most derivatives of six analytes could be separated in the pH range of 3.0–4.0. Consequently, pH 3.5 H_3Cit–Na_2HPO_4 buffer was adopted for further investigation. The concentration of the buffer solution in the mobile phase influences directly the buffer capacity by changing the ionic strength of the solution, which has effect on the separation efficiency. The concentration of H_3Cit–Na_2HPO_4 buffer solution was also discussed in the range of 15–25 mM. Although the retention times of these peaks did not have much difference, better reproducibility and peak shapes were obtained when increasing the buffer concentration. Considering the solubility of the inorganic salt in the mobile phase, 20 mM H_3Cit–Na_2HPO_4 buffer was selected as the optimal. Finally, 20 mM pH 3.5 H_3Cit–Na_2HPO_4 buffer was used in following study since all the derivatives of six analytes could be separated.

Using the above optimized conditions, the separation of six derivatives needed more than 2 h, which resulted in time-consuming separation and peak tailing for late-eluted derivatives. Accordingly, gradient elution was necessary. Moreover, amino compounds coexisting in biological samples also can react with the TMBB-Su reagent, so their interferences should be taken into consideration when optimizing separation conditions. It was found that aliphatic amines and biogenic amines had no interference with the determination of the analytes of interest because of the obvious difference in the polarity between these amino compounds and catecholamines. However, the polarities of amino acids are similar to the analytes. To avoid interferences from these amino acids in biological samples, the chromatographic behaviors of primary amino acid derivatives were investigated to increase the selectivity of the method, including glutamic acid, glycine, glutamine, taurine, aspartic acid, valine, leucine, isoleucine, tryptophan, serine, cysteine, asparagines, threonine, lysine, arginine, histidine, alanine, phenylalanine, proline, γ-aminobutyric acid and glutathione. Through careful and repeated experiments, the optimized gradient elution conditions were obtained as follows. Eluent A was methanol and eluent B was 20 mM pH 3.5 H_3Cit–Na_2HPO_4 buffer solution. The gradient elution condition began with an isocratic elution of 58% A (v/v) for 30 min, followed by a gradual increase of A from 65% to 100% (v/v), until 42 min. Under the optimum conditions, a good separation of

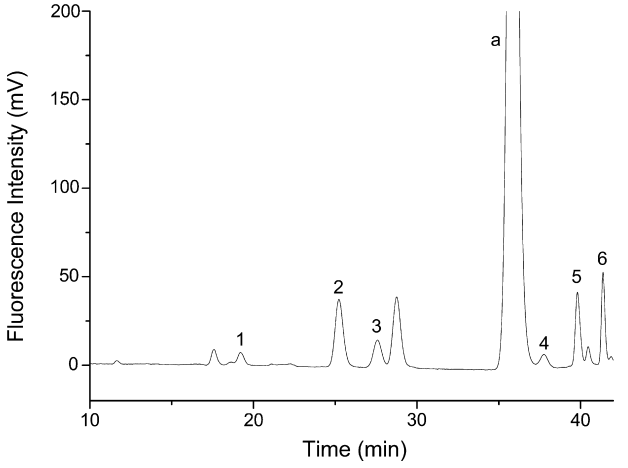

Figure 2 Typical chromatogram of derivatives of the six selected catecholamines and related compounds. Mobile phase: methanol and 20 mM pH 3.5 H_3Cit–Na_2HPO_4 buffer, as gradient program described in Section 2.4. Detection: fluorescence (490/510 nm). Flow rate: 0.7 mL/min. Injection volume: 20 μL. Concentration: 0.05 μM each (L-DOPA concentration: 0.1 μM). Peaks: (1) L-DOPA; (2) Tyr; (3) NE; (4) E; (5) DA; (6) MN and (a) TMBB-Su hydrolyzate.

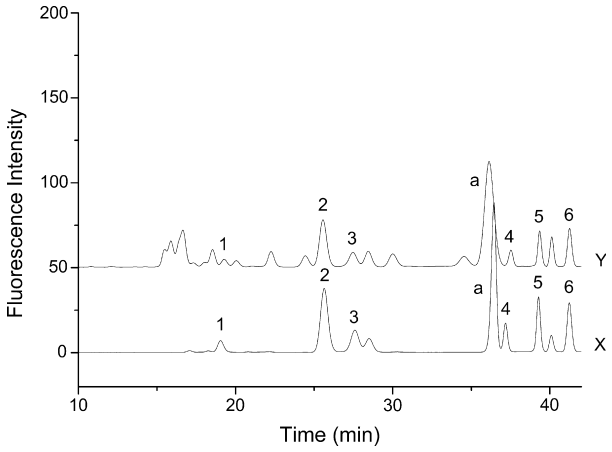

Figure 3 Chromatograms of some interferences on the separation of derivatives of catecholamines and related compounds. X: Chromatogram of the derivatives of six analytes; Y: Chromatogram of the derivatives of six analytes spiked with all amino acids mentioned above and glutathione. Peaks: (1) L-DOPA; (2) Tyr; (3) NE; (4) E; (5) DA; (6) MN and (a) TMBB-Su hydrolyzate.

six derivatives of catecholamines and related compounds was achieved within 42 min (Fig. 2). As shown in Fig. 3, under these conditions, the coexisting amino acids cannot interfere with the determination of catecholamines and related compounds.

3.2. Optimization of derivatization condition to improve the sensitivity

Catecholamines and related compounds have detectable ultraviolet (UV) adsorption, and UV detection is an important approach for their determination in HPLC (Jabbar and Muslih, 2010; Muzzi et al., 2008; Zhang et al., 2003). However, the sensitivity of UV detection is not satisfactory for the quantification of catecholamines and related compounds in clinical and biological samples because of the low concentration of these analytes in these samples. Thus, labeling by fluorescent reagent is a good choice to overcome such drawbacks. To get the best derivatization efficiency, the derivatization conditions have to be optimized. TMBB-Su is a labeling reagent for the amino group and has many advantages like pH and solvent independence, high fluorescence quantum yields and so on (Zhang et al., 2011). According to our previous work, the factors affecting derivatization efficiency between TMBB-Su and amino groups mainly include TMBB-Su concentration, buffer, reaction temperature and reaction time.

The influence of TMBB-Su concentration on reaction efficiency evaluated with the peak areas of derivatives was studied first. *N*-Hydroxysuccinimidyl ester usually can react with amino group selectively in alkaline solution, while, it also hydrolyzes in the same condition. Thus, excessive reagent is desired. To ensure the yield of the derivatization, TMBB-Su concentrations were optimized in the range of 3.5×10^{-4}–7.0×10^{-4} M. As presented in Fig. 4A, the peak areas of the derivatives increase with the increase of the reagent concentration. When TMBB-Su concentration reaches to 6.5×10^{-4} M, almost the peak areas of all derivatives achieve the maximum. Increasing TMBB-Su concentration further has no significant effect on the derivatization yield. Therefore, 6.5×10^{-4} M TMBB-Su was chosen to use in the following experiments.

The effect on derivatization efficiency of the pH value was investigated using H_3BO_3–$Na_2B_4O_7$ buffer solution in detail. As shown in Fig. 4B, the peak areas of all the derivatives alter slightly in the pH range of 7.2–7.8 and pH 7.6 is the most optimum pH value. Moreover, the buffer content was also studied in the range of 5–50 mM. Data obtained suggest that derivatization efficiencies of all the analytes reach the plateau when the buffer content increases to 25 mM, and the peak areas almost keep unchanged when the buffer content is higher than 25 mM. Thus, the derivatization was performed in 25 mM pH 7.6 H_3BO_3–$Na_2B_4O_7$ buffer.

Derivatization temperature affects reaction rate directly. High temperature can accelerate not only the rate of derivatization reaction but also the rate of hydrolysis reaction, so the change of temperature influences greatly the balance of derivatization reaction and results in difference of derivatization efficiency further. As shown in Fig. 4C, although the peak areas of the derivatives of L-DOPA, NE and E are slightly downward and those of the derivatives of Tyr and MN have a subtle increase with the increase of temperature, derivatization temperatures changing from 25 °C to 45 °C have no obvious influence on the peak areas of the derivatives. Considering that it was easier to operate at room temperature, 25 °C was chosen as the final optimum reaction temperature. Derivatization time from 10 min to 50 min at 25 °C was investigated further (Fig. 4D). It can be seen that peak areas of all derivatives reach maximum when derivatization reaction is performed for 40 min at 25 °C. Continuing to prolong reaction time, the changes of peak areas of the derivatives are less than 5%. Finally, the derivatization reaction was chosen to perform at 25 °C for 40 min.

In short, the derivatization reaction of TMBB-Su with the analytes of interest was carried out at 25 °C for 40 min in the presence of 6.5×10^{-4} M TMBB-Su and 25 mM pH 7.6 H_3BO_3–$Na_2B_4O_7$ buffer solution.

3.3. Stability of TMBB-Su and its derivatives

Stability of TMBB-Su was estimated and the results showed that an anhydrous acetonitrile solution of TMBB-Su could be preserved for at least 2 weeks stably at 4 °C. Although catechols are not stable enough at alkaline medium and are easily oxidized to quinines (Chan et al., 2000), the derivatization reaction was carried out in weakly alkaline solution (pH 7.6) in our experiment, which keeps catechols from oxidation as much as possible in the derivatization process. Stability of the formed derivatives was also examined by the analysis of the change in the peak areas and it was found that the derivatives were stable with the variations of peak areas within 4.0% (n = 6) for at least three days at 4 °C or one day at room temperature.

3.4. Analytical performance

The linear calibrations were obtained by determining mixtures of catecholamines and related compounds with seven different concentrations under the optimized separation and derivatization conditions as described above. Liner calibration range,

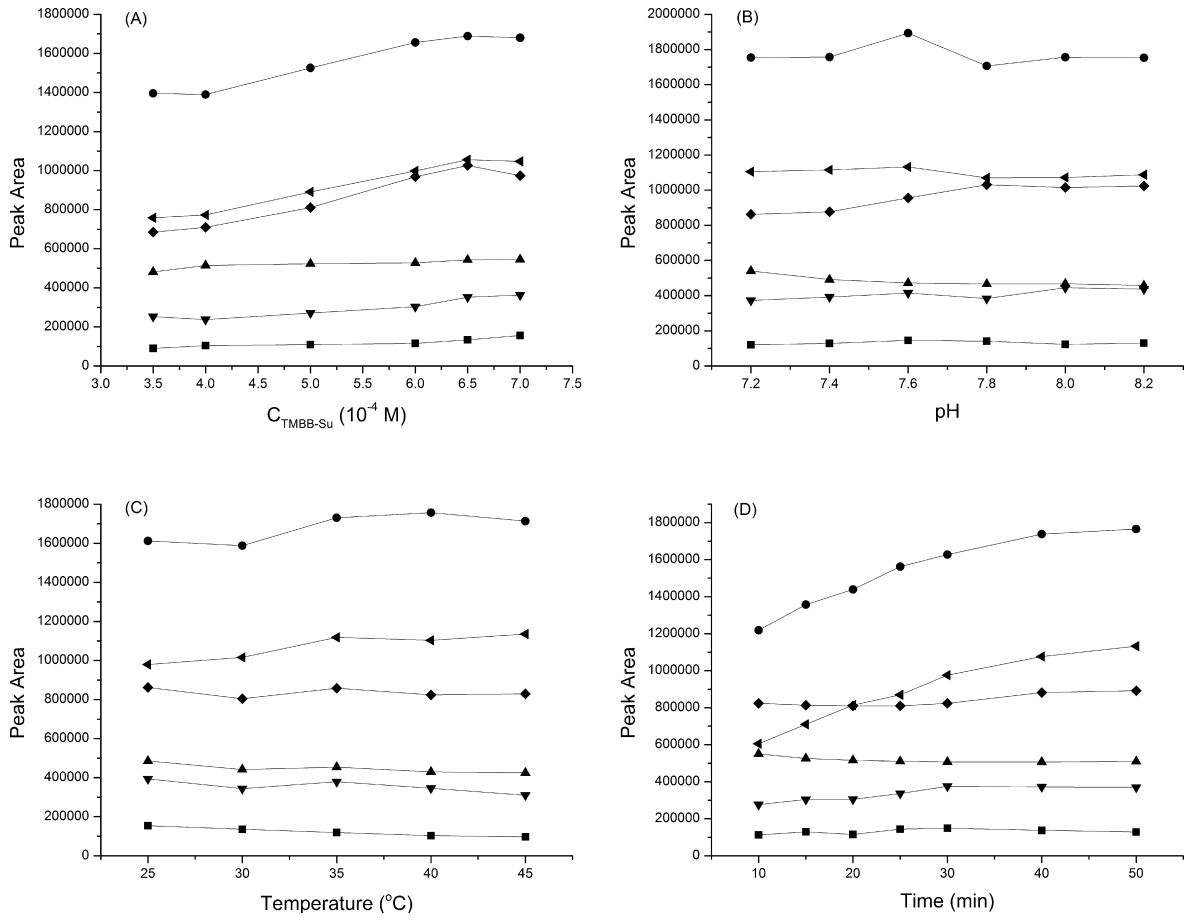

Figure 4 Optimization of derivatization conditions. Effect of (A) TMBB-Su concentration; (B) buffer pH; (C) reaction temperature; (D) reaction time on peak areas. Symbol assignation: (■) TMBB-L-DOPA; (●) TMBB-Tyr; (▲) TMBB-NE; (▼) TMBB-E; (♦) TMBB-DA; (◄) TMBB-MN.

Table 1 Liner calibration range, regression equation, correlation coefficients square and detection limits of the method.

Analyte	Calibration range (μM)	Regression equation[a]	R^2	Detection limit[b] (nM)
L-DOPA	0.004–0.2	$Y = 1154.4 + 2.964 \times 10^6 X$	0.9986	1.45
Tyr	0.002–0.5	$Y = 11260.0 + 3.317 \times 10^7 X$	0.9992	0.17
NE	0.002–1	$Y = 53487.8 + 1.001 \times 10^7 X$	0.9997	0.40
E	0.002–1	$Y = 28252.8 + 7.570 \times 10^6 X$	0.9995	0.40
DA	0.002–0.5	$Y = 41961.4 + 1.823 \times 10^7 X$	0.9992	0.10
MN	0.002–0.2	$Y = 22938.1 + 2.391 \times 10^7 X$	0.9981	0.10

[a] X: concentration of analytes (μM); Y: peak area of analytes derivatives.
[b] $S/N = 3$.

regression equation, correlation coefficients square and detection limits are summarized in Table 1. Linear ranges are from 0.004 to 0.2 μM for L-DOPA, from 0.002 to 0.5 μM for Tyr and DA, from 0.002 to 1 μM for NE and E and from 0.002 to 0.2 μM for MN, respectively. The correlation coefficients square (R^2) is between 0.9981 and 0.9997, which displays good linearities in the corresponding concentration ranges. The detection limits ($S/N = 3$) for the labeled catecholamines and related compounds are in the range of 0.10–0.40 nM (L-DOPA: 1.45 nM), which are sufficiently sensitive for the determination of catecholamines and related compounds in biological samples. The precision of the method expressed as the relative standard deviation (RSD) is presented in Table 2. RSDs are from 0.7% to 3.7% for intra-day determination ($n = 6$) and from 1.4% to 4.8% for inter-day determination ($n = 6$).

The comparison of the proposed method and existing HPLC methods based on fluorescence derivatization for the detection of catecholamines and related compounds is presented in Table 3. Longer detection wavelengths can be seen for TMBB-Su compared with those for other fluorescent reagents listed except SIFA. Moreover, the derivatization conditions including derivatization pH and temperature for TMBB-Su are milder than those for other several reagents,

Table 2 Intra-day and inter-day precision of the proposed method.

Analyte	Concentration (μM)	Intra-day precision; RSD (%), $n = 6$	Inter-day precision; RSD (%), $n = 6$
L-DOPA	0.02	0.9	1.4
	0.1	1.6	2.3
	0.2	1.3	2.0
Tyr	0.01	2.6	3.7
	0.05	0.7	1.5
	0.1	1.2	2.7
NE	0.01	3.0	3.2
	0.05	2.2	3.6
	0.1	1.5	1.6
E	0.01	1.4	2.3
	0.05	3.3	4.8
	0.1	2.0	3.2
DA	0.01	3.7	4.6
	0.05	2.5	3.0
	0.1	3.4	3.8
MN	0.01	1.6	2.0
	0.05	2.8	3.5
	0.1	0.9	1.8

which can avoid the oxidation of phenolic hydroxyl groups of catechols effectively in the derivatization process in the proposed method. It is also observed that the detection limits of the proposed HPLC-FLD approach are lower than those obtained with the existing HPLC methods based on fluorescence derivatization. The advantages of the proposed method are apparent, including relatively long detection wavelengths, mild derivatization conditions and excellent sensitivity, which provide wide applications for the analysis of catecholamines and related compounds in biological samples.

3.5. Sample analysis

Liver is a main metabolic place of many compounds, while the brain is the main target of the functions of catecholamine neurotransmitters. In order to verify the proposed HPLC–FLD method based on derivatization for the simultaneous determination of catecholamines and related compounds (including Tyr, L-DOPA, DA, NE, E and MN), mice livers were used as the representative of samples for metabolic analysis and mice brains for neurotransmitters analysis. Under the optimal derivatization and separation conditions, the chromatograms of the samples and the same samples spiked with standard solutions were obtained as presented in Fig. 5. The concentrations of analytes in biological samples were

Table 3 Comparison of the proposed method and those existing HPLC methods based on fluorescence derivatization for the detection of catecholamines and related compounds.

Reagent	Ex/Em (nm)	Derivatization condition	Detection limit (nM)	Reference
OPA	350/450	pH 10.0, r.t., 2 min (2-mercaptoethanol)	26	(Zhao et al., 2011)
Fluorescamine	390/480	pH 8.0	796	(Díaz et al., 2009)
FMOC-Cl	263/313	pH 8.0, r.t., 15 min	3.75	(Chan et al., 2000)
SIIA	301/365	pH 8.5, 60 °C, 15 min	2.15–9	(Wang et al., 1999)
SIFA	490/516	pH 8.5, 45 °C, 20 min	0.16–2.8	(Wang et al., 2000)
DPE	350/480	pH 9.0, 30 °C, 20 min	1.1–4.7	(Liu et al., 2011)
TMBB-Su	490/510	pH 7.6, r.t., 40 min	0.10–0.40 (L-DOPA: 1.45)	This work

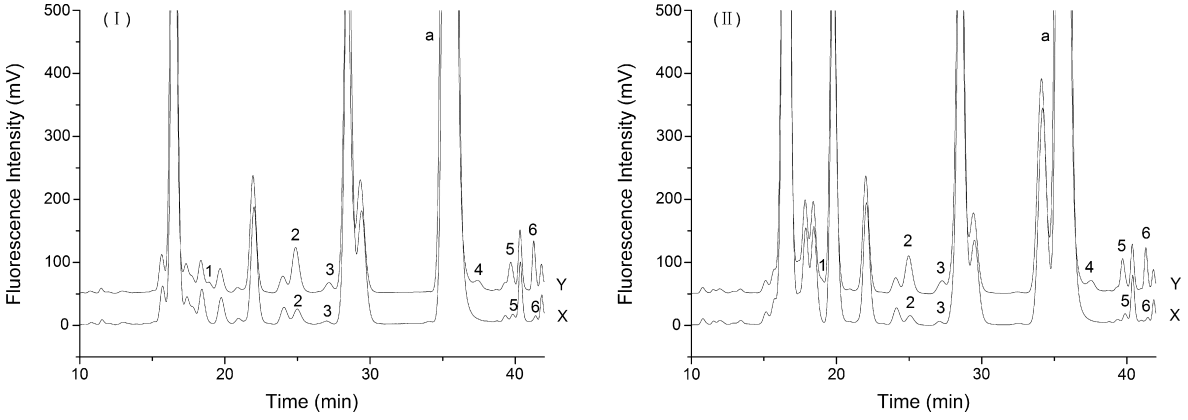

Figure 5 Chromatograms obtained from samples. Peaks: (1) L-DOPA; (2) Tyr; (3) NE; (4) E; (5) DA; (6) MN; (a) TMBB-Su hydrolyzate. (I) Chromatograms obtained from (X) liver sample, and (Y) the same sample spiked with 0.05 μM of standard analytes (L-DOPA: 0.1 μM). (II) Chromatograms obtained from (X) brain sample, and (Y) the same sample spiked with 0.05 μM of standard analytes (L-DOPA: 0.1 μM).

Table 4 Analytical results for the liver and brain samples from mice.

Samples	Liver sample				Brain sample			
	Added (ng/mg)	Founded (ng/mg)	RSD (%, $n = 6$)	Recovery (%)	Added (ng/mg)	Founded (ng/mg)	RSD (%, $n = 6$)	Recovery (%)
L-DOPA	0	0	–	–	–	–	–	–
	56.34	51.65	3.8	91.7	–	–	–	–
	112.68	121.69	2.0	108.0	–	–	–	–
	157.75	145.42	3.4	92.2	–	–	–	–
Tyr	0	26.92	0.8	–	0	15.53	2.3	–
	25.89	54.54	2.2	106.7	25.89	38.50	1.9	88.7
	51.77	81.79	1.1	106.0	51.77	66.26	3.0	98.0
	72.48	105.53	2.7	108.5	72.48	81.76	2.0	91.4
NE	0	13.53	2.5	–	0	15.47	1.0	–
	24.17	38.02	3.5	101.3	24.17	37.04	1.8	89.2
	48.34	59.94	1.7	96.0	48.34	64.77	1.2	102.0
	67.68	86.34	1.6	107.6	67.68	88.10	1.4	107.3
E	0	0	–	–	0	0	–	–
	26.17	25.05	2.8	95.7	26.17	24.09	2.6	92.1
	52.34	53.91	3.4	103.0	52.34	52.03	1.5	99.4
	73.28	79.82	1.4	108.9	73.28	69.63	4.0	95.0
DA	0	2.10	2.6	–	0	2.10	2.8	–
	21.89	24.35	3.2	101.6	21.89	24.57	4.3	102.6
	43.77	48.12	2.5	105.1	43.77	47.94	1.2	104.7
	61.28	67.84	3.8	107.3	61.28	68.26	3.4	108.0
MN	0	3.27	1.3	–	0	1.92	2.2	–
	28.17	29.08	2.0	91.6	28.17	27.76	1.3	91.7
	56.34	57.24	1.8	95.8	56.34	54.33	1.7	93.0
	78.88	89.02	4.3	108.7	78.88	72.20	2.0	89.1

calculated according to the corresponding linear regression equations and peak areas. The accuracy of the proposed method was evaluated with the recovery. The analytical results of samples are listed in Table 4. Tyr, NE, DA and MN are detected in the samples. The levels in mice brains are 15.53 ng/mg for Tyr, 15.47 ng/mg for NE, 2.10 ng/mg for DA and 1.92 ng/mg for MN, respectively. And the levels of catecholamines DA and NE in mice brains obtained are in accordance with data reported in other literatures (Felice et al., 1978; González et al., 2011; Mu et al., 2014). The recoveries of all the analytes in samples range from 88.7% to 108.9% and RSDs vary from 0.8% to 4.3%.

4. Conclusions

This work developed a simple, convenient and highly sensitive method for the simultaneous determination of catecholamines and related compounds including Tyr, L-DOPA, DA, NE, E and MN in one whole metabolic pathway using HPLC–FLD based on TMBB-Su derivatization. Meantime, as an exploration of the determination of metabolites in the metabolic network of catecholamines, the proposed method provides a new alternative for the metabolic study of catecholamines. The detection limits obtained are 0.10–0.40 nM except L-DOPA (1.45 nM). Moreover, the proposed method has been applied for the analysis of mice liver and brain samples, which indicates that the proposed method is versatile for the complex sample analysis of catecholamines and related compounds in both metabolic study and neurotransmitter study.

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